ELSEVIER

Synaptic development: insights from *Drosophila*

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In *Drosophila*, the larval neuromuscular junction is particularly tractable for studying how synapses develop and function. In contrast to vertebrate central synapses, each presynaptic motor neuron and postsynaptic muscle cell is unique and identifiable, and the wiring circuit is invariant. Thus, the full power of *Drosophila* genetics can be brought to bear on a single, reproducibly identifiable, synaptic terminal. Each individual neuromuscular junction encompasses hundreds of synaptic neurotransmitter release sites housed in a chain of synaptic boutons. Recent advances have increased our understanding of the mechanisms that shape the development of both individual synapses — that is, the transmitter release sites including active zones and their apposed glutamate receptor clusters — and the whole synaptic terminal that connects a pre- and post-synaptic cell.

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Introduction

The synapse is the primary site of communication between neurons and the fundamental unit of function in the nervous system. Synapse formation involves bidirectional signals between pre- and post-synaptic cells that lead to the development of specialized structures for the release and detection of neurotransmitter. Whereas the basic mechanism of synapse formation is probably genetically determined, the function, maturation and stability of synapses are dynamically regulated during development. Such developmental plasticity not only underlies the refinement of neural circuits, but similar mechanisms might function throughout life to mediate activity-dependent synaptic change. As such, identifying molecular pathways that shape synapse development is a central goal of the developmental neurobiologist. In this review, we describe recent work from the *Drosophila* neuromuscular junction (NMJ) that provides insights into synaptic development.

The *Drosophila* NMJ as a model synapse

The *Drosophila* NMJ is a favorite model system for studies of the synapse. First, it has the advantage of the power and elegance of modern *Drosophila* genetics. In addition to the obvious benefits of generating and analyzing mutants, synaptic studies are greatly aided by the ability to control gene function in a temporal and tissue-restricted manner. Such techniques facilitate the precise manipulation of circuits, including the differential regulation of gene function in adjacent target cells of a single motor neuron for the study of synaptic competition.

Second, the NMJ is accessible to various experimental techniques. Electrophysiology, FM (frequency-modulated) dye labeling, Ca^{2+} imaging and behavioral studies all probe the physiological function of the synapse. Immunohistochemistry, electron microscopy and live imaging provide a clear view of the structural and molecular anatomy of the synapse.

Third, the *Drosophila* NMJ is glutamatergic. As a result, its molecular constituents — and potentially its developmental mechanisms — resemble vertebrate, central glutamatergic synapses more closely than does the vertebrate cholinergic NMJ.

Fourth, each *Drosophila* NMJ is unique and identifiable. In each segmental unit of the neuromuscular system, 32 identified motor neurons synapse with 30 identified postsynaptic muscle cells in a stereotyped pattern. Not only are the cells stereotyped, but the arborization pattern and synaptic strength are also roughly stereotyped. Thus, multiple iterations of an identified NMJ can be analyzed in a single fly, and that same NMJ can be reliably compared from fly to fly. This single-synapse resolution enables subtle changes to be observed and characterized when examining mutants.

Last, despite its stereotyped circuitry, the *Drosophila* NMJ shows robust plasticity. Changes in the environment, neuronal activity and gene function all lead to modification of synaptic structure and function during development. Thus, the *Drosophila* NMJ combines many of the best features of the simple, genetically tractable *Caenorhabditis elegans* model with the more complex, physiologically accessible mouse model.

Before describing recent advances, we wish to highlight an important semantic issue. Researchers in the field

(including us) have an unfortunate tendency to use the term 'synapse' to describe two very different structures. First, the whole synaptic connection formed between a motor neuron and muscle is often referred to as a synapse. Such a structure comprises a branched chain of synaptic boutons formed by the motor neuron and typically surrounded by an elaborate membranous compartment made by the muscle. For an average NMJ, this connection can include 20–50 synaptic boutons. Within each bouton, however, are multiple presynaptic release sites, termed active zones, where synaptic vesicles cluster and fuse. Opposite each active zone, postsynaptic glutamate receptors cluster to sense the released transmitter. This dyad of active zone and glutamate receptor cluster is also called a synapse, and is more akin to the usual definition of vertebrate glutamatergic synapses. Because each bouton contains approximately ten active zones, each motor neuron can form upwards of 500 such synapses with a single postsynaptic cell.

We highlight this semantic point because the development of each structure is probably controlled by very different molecular mechanisms. As such, we address each structure separately in this review. We refer to the chain of boutons formed between motor neuron and muscle as the 'synaptic terminal', and reserve the term 'synapse' for individual active zones and their apposed receptor cluster.

Synapse development

Active zones at the synapse

For many years, studies of motor neuron morphology at the NMJ have relied on antibodies that recognize neuronal membrane proteins, the neuronal cytoskeleton and/or synaptic vesicle proteins. By using these reagents, numerous mutants have been characterized that alter synaptic terminal morphology. These tools are inadequate, however, for studies of individual synapses at the NMJ. Recently, the identification of the first active-zone protein in *Drosophila*, Bruchpilot (BRP), along with the characterization of a monoclonal antibody that recognizes BRP, has opened up the study of active-zone development and function in the fly [1••,2•].

BRP is the *Drosophila* homolog of the vertebrate active-zone protein ELKS (also known as CAST and ERC). ELKS is a constituent of the vertebrate active zone and binds to a large complex containing the active-zone proteins Munc-13, RIM1, Piccolo and Bassoon. In *Drosophila*, BRP localization has been characterized by sub-diffraction resolution stimulated emission depletion (STED) fluorescence microscopy [1••]. BRP is present in donut-shaped structures that surround and delineate each active zone. In the fly, many active zones contain electron-dense specializations known as 'T-bars', which probably promote vesicular transmitter release. In *brp* mutants, electron microscopy reveals tight apposition of pre- and post-synaptic membranes characteristic of active zones; however, T-bars are absent and Ca^{2+} channel expression is markedly reduced at the synapse. Thus, BRP is required for the development of normal active zones. These aberrant active zones do not function well: after an action potential, fewer synaptic vesicles are released and their fusion is asynchronous. By controlling localization of T-bars and Ca^{2+} channels to the active zone, BRP is ideally situated to regulate synaptic strength at individual release sites.

Glutamate receptors at the synapse

Glutamate receptors at the *Drosophila* NMJ comprise two types depending on which variable subunit, DGluRIIA or DGluRIIB, they contain. The other known subunits, DGluRIII, DGluRIID and DGluRIIE, are probably present in all ionotropic glutamate receptors (iGluRs) [3–5]. Receptors containing DGluRIIA or DGluRIIB differ in their single channel properties, synaptic responses, regulation by second messengers and localization [6].

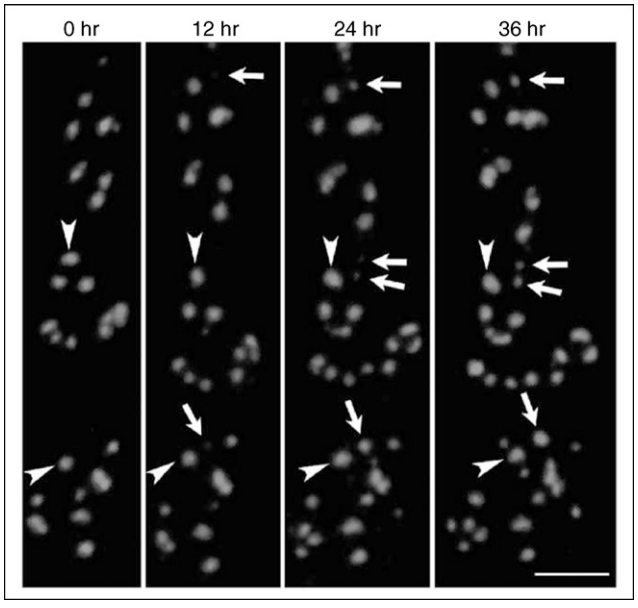
With the structural subunits of these synaptic receptors identified, attention is turning to the mechanisms by which the nerve induces clustering of iGluRs during synapse formation. Studies have shown that presynaptic activity is required for glutamate receptor clustering [7], possibly owing to a requirement for spontaneous vesicle fusion, although this finding is controversial [8,9]. Release of glutamate itself is not required for glutamate receptor clustering, because mutants in the *Drosophila* vesicular glutamate transporter do not release vesicular glutamate but receptors still cluster [10]. In fact, non-vesicular glutamate negatively regulates receptor clustering, potentially through a ligand-induced internalization mechanism [8]. Several postsynaptic proteins have been identified that regulate the extent of glutamate receptor clustering [11–14], but no molecules other than the receptors themselves are absolutely required for receptor clustering. An outstanding mystery in the field is the identity of the putative trans-synaptic signaling pathway analogous to agrin at the vertebrate NMJ that initiates postsynaptic differentiation and subsequent synaptic localization of glutamate receptors.

Live imaging of glutamate receptor clustering

Molecular factors regulating glutamate receptor localization continue to be defined, but a deep understanding of the mechanism of receptor clustering requires a detailed description of the cell biology of the system. Towards that end, a landmark study has described glutamate receptor dynamics during synapse formation by using *in vivo*, live imaging techniques [15••].

Rasse *et al.* [15••] generated a DGluRIIA transgene tagged with green fluorescence protein that is functional and rescues the DGluRIIA mutant. New receptor puncta were not observed to split from pre-existing receptor clusters. Instead, the tagged receptor forms clusters at

Figure 1



In vivo imaging of glutamate receptor clustering. Larvae expressing green fluorescence protein-tagged DGluRIIA were imaged at the indicated time points. This *in vivo* analysis reveals that some receptor clusters are stable (arrowheads), whereas others are newly formed (arrows). Newly formed receptor clusters reach their mature size in ~24 h. Scale bar represents 4 μm. Figures are reproduced with permission from [15••].

newly formed postsynaptic densities that grow to a mature size in about 24 h (Figure 1). This postsynaptic specialization precedes the development of presynaptic active zones, as defined by staining for the active-zone marker BRP [2•], by at least 3 h. Receptors enter newly forming clusters from a diffuse, cell-wide pool of receptors. Whereas the clustered receptors are largely immobilized, other postsynaptic proteins such as the scaffolding protein Discs-large, the adhesion molecule Fasciclin II, and the kinase Pak move more freely into and out of the synapse. As more proteins become accessible to this live imaging approach, we can expect important insights into the differential regulation of glutamate receptor subtypes and the coordination of pre- and post-synaptic development at the level of individual release sites.

Apposition of active zones and glutamate receptors

Identification of the constituents of active zones and receptor clusters, coupled with the development of reagents to visualize these structures by light microscopy, creates an exciting opportunity for analyzing synapse development. Live imaging studies should define the sequence of events underlying synapse formation, whereas a new wave of genetic screens will identify genes that are necessary for the development, maturation and maintenance of the complex of active zones and receptor clusters at the synapse.

The mechanisms that coordinate the development of this trans-synaptic complex are excellent candidate substrates for plasticity mechanisms. In fact, a recent study suggests that each synapse might be differentially regulated during synaptic development. At each NMJ, glutamate receptors expressed in a single postsynaptic cell are confronted by an array of hundreds of apposed active zones, each with different morphological and physiological properties. When receptor is limiting, glutamate receptors do not distribute uniformly opposite active zones. Instead, they preferentially localize to the active zones that are larger and have a higher probability of neurotransmitter release than the average active zone [16]. Putting more receptors opposite to these high-probability release sites maximizes synaptic strength, and suggests a mechanism for activity-dependent matching of pre- and post-synaptic function at the level of single active zones.

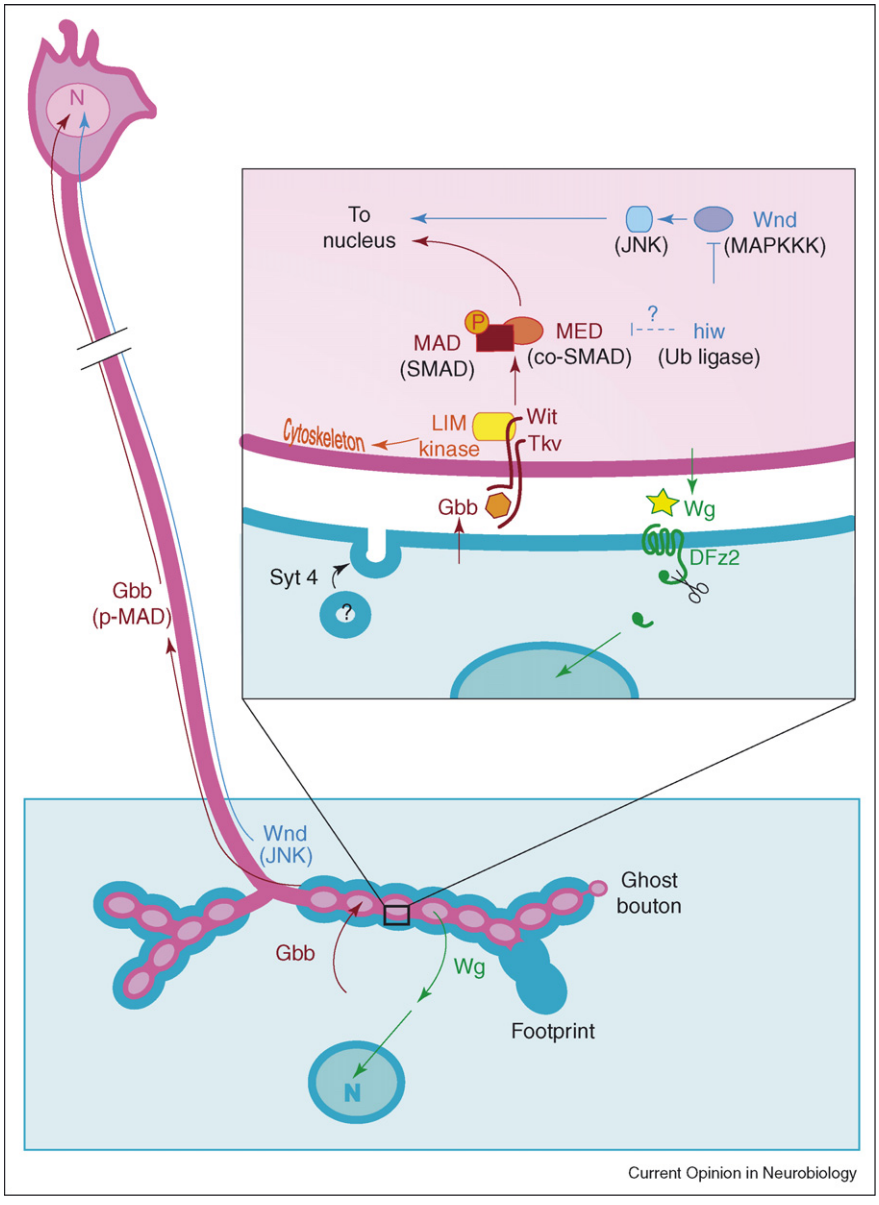
Synaptic terminal development

Trans-synaptic signals control synaptic terminal development

The synaptic terminal encompasses all of the synaptic contacts between a motor neuron and postsynaptic muscle cell. Development of the *Drosophila* NMJ synaptic terminal is regulated by trans-synaptic signals, including Wingless (Wg), a member of the Wnt family, and Glass bottom boat (Gbb), a member of the bone morphogenetic protein (BMP) and transforming growth factor-β (TGFβ) family [17]. At the NMJ, Wg and Gbb act like classical morphogens, stimulating a signal to the nucleus that presumably alters gene expression in the receiving cell (Figure 2). For Wg, however, the signal transduction mechanism at the synapse can differ greatly from those that function during embryonic patterning. At the *Drosophila* NMJ, the Wg ligand is secreted by the motor neuron and endocytosed into the postsynaptic muscle, where it probably acts as an anterograde signal [18]. Disrupting Wg signaling results in marked defects in synaptic nerve terminal morphology — some boutons lack active zones and mitochondria, and postsynaptic proteins including glutamate receptors are delocalized. Similar defects in nerve terminal morphology occur in mutants of the Wg receptor, DFrizzled2 (DFz2) [19••,20]. Because these phenotypes are rescued by postsynaptic expression of DFz2, the Wg signal transduction pathway functions in the muscle and might influence presynaptic morphology through an unknown retrograde mechanism.

The transduction pathway in muscle is being defined and is remarkably distinct from the previously described canonical and non-canonical pathways of Wg signaling [19••,20]. In muscle, Wg stimulates the proteolytic cleavage of a DFz2 intracellular domain, liberating a C-terminal fragment of 8 kDa that translocates to the nucleus (Figure 2). This 8-kDa DFz2 fragment localizes to actively transcribed regions of chromosomes, although it is not known whether it regulates gene expression.

Figure 2



Signaling at the synaptic terminal. During development, the motor neuron (pink) grows chains of synaptic boutons to innervate a muscle (light blue). Several pathways are known to regulate the formation and stability of the synaptic terminal structure. These include a Wg pathway (green), which signals from the neuron to the muscle, and the TGFβ/BMP ligand Gbb (orange), which signals from muscle to the motor neuron. Wg signaling proceeds through a recently described mechanism in which the Wg receptor DFz2 is cleaved, liberating a small C-terminal DFz2 fragment that translocates into the muscle nucleus (N) [19••]. Gbb signaling proceeds through the receptors Wit (type II), Tkv (type I) and Sax (another type I, not shown). Gbb stimulates phosphorylation of the downstream effector MAD, which, with the assistance of co-SMAD Medea (MED), translocates into the motor neuron nucleus. The Gbb pathway was previously proposed to be regulated by the ubiquitin ligase Highwire (hiw) [48], whose absence causes marked overgrowth of the synaptic terminal. Recent findings suggest, however, that Highwire regulates synaptic terminal structure by targeting the Wallenda (Wnd) MAPKKK [49••]. Wallenda signals through JNK and the Fos transcription factor, and thus is also likely to regulate a signal to the motor neuron nucleus. Because the Wallenda signal does not require the Jun transcription factor, it is likely to mediate a JNK signal that is distinct from a previously described JNK signal [62], which, along with cAMP signaling [63], has not been shown here owing to space constraints. Syt4 regulates the secretion of a retrograde signal, the identity of which is currently unknown. The Gbb pathway, along with LIM kinase, a regulator of cytoskeleton, has been recently implicated in regulating the stability of the synaptic terminal structure [34••] by influencing the appearance of synaptic 'footprints' — sites that contain postsynaptic structure (blue) with no apposing presynaptic structure (pink). Conversely, disruption of Wg signaling results in the appearance of 'ghost boutons', which have no apposing postsynaptic structure.

Although this fragment is not sufficient for Wg signaling, it might be necessary: rescue experiments show that mutations impairing DFz2 cleavage disrupt Wg signaling [19••]. Because the region around the cleavage site of DFz2 is conserved in vertebrate Frizzled homologs, this mechanism might be evolutionarily conserved.

Complementary to the anterograde Wg signal, Gbb mediates a retrograde signal from the muscle to the motor neuron. This pathway has been recently reviewed [17,21], and we summarize it only briefly here. Gbb released from muscle stimulates the BMP/TGFβ receptors Wit, Tkv and Sax in the presynaptic motor neuron to phosphorylate the downstream effector MAD. Phosphorylated MAD, with assistance from the co-SMAD Medea, translocates to the motor neuron nucleus (Figure 2). Because the mutant phenotype for disrupting this pathway — namely, a marked reduction in the number of boutons — correlates with an absence of phosphorylated MAD in the nucleus, this pathway probably regulates gene expression, but the targets of regulation are currently unknown. Components of the pathway, including Tkv and phosphorylated MAD, are also abundant at the postsynaptic membrane [22], although no postsynaptic function has been explicitly described.

A recent study has identified Syt4, a member of the synaptotagmin family expressed in the muscle, as a candidate regulator of a trans-synaptic, retrograde signal [23••] (Figure 2). When Syt4 is overexpressed in only one of two muscles that are innervated by the same motor neuron, the motor neuron grows extra boutons only on the muscle that overexpresses Syt4. Although the identity of the retrograde signal or signals at work here remains to be determined, this finding builds on an interesting previous observation that nerve terminals from a single motor neuron respond differentially to target-derived cues [24].

Whereas the Gbb and Wg pathways signal to the nucleus, regulation of nerve terminal structure is ultimately mediated through local changes to cytoskeleton and membrane. Several studies have begun to investigate the contribution of cytoskeletal proteins to synaptic terminal structure [25–33,34••]. Important factors in regulating local changes probably include the receptor tyrosine phosphatase D-LAR and its newly identified extracellular ligands [35•,36•,37], adhesion molecules such as Fasciclin II [38], and regulators of membrane trafficking [39–42].

Restraining synaptic terminal growth

Synaptic terminal development requires factors that promote growth, such as Wg and Gbb, in addition to mechanisms that restrain growth. The most potent negative regulator of synaptic terminal growth identified to date is *highwire*: in its absence, the terminal undergoes excessive growth, forming a huge excess of synaptic boutons and branches [43]. *highwire* encodes an extremely large (566 kDa) evolutionarily conserved molecule. Studies in *Drosophila*, *C. elegans* and zebrafish suggest that Highwire functions as an E3 ubiquitin ligase, probably targeting a synaptogenic protein for degradation [44•,45–47]. Because the Gbb signal mediates synaptic terminal growth, its signaling pathway is an attractive candidate for regulation by Highwire, and genetic and physical interactions that support this model have been observed. Highwire can bind the BMP downstream effector SMAD Medea, and mutations in components of the Gbb–Wit pathway partially suppress the *highwire* overgrowth phenotype [48].

In contrast to the partial suppression by the Gbb–Wit pathway, work in our laboratory has recently identified a mutant that can completely suppress the *highwire* overgrowth phenotype [49••]. This mutant disrupts Wallenda, a mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK) with homologs in vertebrates and *C. elegans*. Wallenda behaves in all respects as a functionally relevant target of Highwire. Levels of Wallenda protein are markedly increased in a *highwire* mutant, and overexpression of Wallenda is sufficient to cause synaptic terminal overgrowth. Furthermore, work in *C. elegans* has identified an identical relationship between the worm homologs of Highwire and Wallenda [50]; thus, the regulation of this MAPKKK by Highwire is an evolutionarily conserved mechanism. In *Drosophila*, Wallenda signals through the JNK MAPK and the transcription factor Fos, suggesting that this pathway regulates transcriptional events that influence synaptic terminal growth [49••] (Figure 2). By contrast, Highwire does not influence the levels of phosphorylated MAD (an effector of BMP signaling) in the motor neuron nucleus, and there is currently no evidence that Highwire regulates the levels of any components of the Gbb pathway. Although Highwire might regulate both pathways, it is also possible that Highwire–Wallenda and Gbb–Wit are parallel pathways that each modulate synaptic growth.

The regulation of Wallenda levels by an E3 ubiquitin ligase exemplifies the important role of ubiquitination for synaptic development (reviewed most recently in [51]). Indeed, the local control of protein degradation and synthesis might be particularly important at the synapse, which is far from the cell body. A series of studies indicates that regulated protein synthesis and turnover are key modulators of synaptic terminal development and function at the *Drosophila* NMJ [52–57].

Synaptic terminal stability

Synaptic terminal morphology is shaped by both the maintenance and the formation of synaptic contacts. Indeed, many developing neuronal circuits initially form exuberant synaptic contacts that are later refined by selective elimination and stabilization [58]. At the *Drosophila* larval NMJ, synapse elimination does not

regulate connectivity as it does at the vertebrate NMJ. Synaptic boutons can, however, be lost during development. This loss is visualized as a 'footprint' — that is, a bouton or branch of boutons that stain for postsynaptic markers but have no apposed presynaptic structure [59] (Figure 2). Because the pre- and post-synaptic structures develop in tandem, the footprints imply that boutons were present and have since retracted. Mutants with excess footprints implicate various processes in the regulation of synaptic stability, including axon transport [59], the cytoskeleton [60] and, interestingly, the Gbb–Wit signaling pathway, which seems to influence synaptic stability locally through interactions with LIM kinase, a modulator of the cytoskeleton [34••].

Synaptic terminal loss might also involve the disassembly of postsynaptic elements, resulting in presynaptic terminals that are not apposed to postsynaptic specializations. This phenotype has been observed in mutants that disrupt Wg signaling, and the presynaptic elements are called 'ghost' boutons [20] (Figure 2). This phenotype might reflect a requirement for Wg signaling in maintenance of a stable synaptic structure, but it might also reflect a defect in bouton formation. Indeed, analysis of synaptic stability will greatly benefit from live imaging techniques that can disentangle defects in formation from maintenance. Lastly, analysis of synaptic stability might be better performed during development of the adult NMJ, where marked retraction and elimination events are necessary for establishing the final adult structure [61].

Conclusions

Analysis of synaptic development at the *Drosophila* NMJ has matured in recent years. The traditional metric for analyzing NMJ development has been to count the number of synaptic boutons. Although this is still an important descriptor of a developing synapse, as a single measure it is inadequate. As our understanding of cell biological and signal transduction mechanisms grows, novel genes that shape synaptic development should be placed within the rich context of developmental mechanisms at work at the NMJ. Does a new mutant with fewer boutons inhibit BMP signaling to the nucleus, disrupt the synaptic cytoskeleton, enhance synaptic retraction, or define a novel synaptic mechanism?

Answering such questions is the first step towards integrating our knowledge of individual molecules at the synapse into a broader explanatory framework for how the neuromuscular system develops. Such a deep understanding of developmental mechanisms not only will give insights into how synapses form, but also might reveal mechanisms operating during activity-dependent structural synaptic plasticity. Knowledge generated at the *Drosophila* NMJ will provide reagents and hypotheses for studying neuronal synapses in the *Drosophila* brain. Finally, every molecule mentioned in this review has a homolog expressed in the mammalian brain. Insights gleaned from *Drosophila* should allow our understanding of mammalian synaptic development to fly to new heights.

Update

Three recent papers provide additional insights into the molecular mechanisms underlying synaptic development. Pielage *et al.* demonstrate that the selective disruption of postsynaptic spectrin leads to abnormally large active zones whose spacing is altered. They propose that a postsynaptic spectrin–actin lattice acts as a scaffold to organize pre- and post-synaptic development [64]. Work from Schmid *et al.* investigates the role of ionotropic glutamate receptors for synaptic development [65]. They find that, in the absence of postsynaptic transmitter receptor, synapse formation occurs normally but synapse maturation is impaired. Synapse maturation does not require synaptic transmission or glutamate binding to the glutamate receptors, suggesting that the receptors play a structural role during development [65]. Martín-Peña *et al.* find that PI3 kinase signaling influences the number of synaptic contacts. Increasing PI3 kinase activity can induce the formation of extra pre-synaptic active zones and increase synaptic activity [66]. This study also suggests that PI3 kinase signaling can influence synapse formation and maintenance in the adult brain.

For a more detailed review of methodologies and insights from work using the *Drosophila* NMJ, we recommend [67].

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- • of special interest
- •• of outstanding interest

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